

# The Entry of Diphtheria Toxin into the Mammalian Cell Cytoplasm: Evidence for Lysosomal Involvement

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**ABSTRACT** Lysosomotropic amines, such as ammonium chloride, are known to protect cells from the cytotoxic effects of diphtheria toxin. These drugs are believed to inhibit the transport of the toxin from a receptor at the cell exterior into the cytoplasm where a fragment of the toxin arrests protein synthesis. We studied the effects of lysosomotropic agents on the cytotoxic process to better understand how the toxin enters the cytoplasm.

The cytotoxic effects of diphtheria toxin were not inhibited by antitoxin when cells were preincubated at 37°C with toxin and ammonium chloride, exposed to antitoxin at 4°C, washed to relieve the ammonium chloride inhibition, and finally warmed to 37°C. The antigenic determinants of the toxin were, therefore, either altered or sheltered. It is likely that the combination of ammonium chloride and a low temperature trapped the toxin in an intracellular vesicle from which the toxin could proceed to the cytoplasm. Because lysosomotropic amines raise the pH within acidic intracellular vesicles, such as lysosomes, they could trap the toxin within such a vesicle if an acidic environment were necessary for the toxin to penetrate into the cytoplasm. We simulated acidic conditions which the toxin might encounter by exposing cells with toxin bound to their surface to acidic medium. We then measured the effects of lysosomotropic amines on the activity of the toxin to see if the acidic environment substituted for the function normally inhibited by the drugs. The drugs no longer protected the cells. This suggests that exposing the toxin to an acidic environment, such as that found within lysosomes, is an important step in the penetration of diphtheria toxin into the cytoplasm.

Diphtheria toxin is a prokaryotic protein (63,000 mol wt) that arrests protein synthesis in most mammalian cells. The first step in this process occurs at the cell surface where the toxin binds to a receptor. The last step occurs in the cytoplasm where fragment A (21,000 mol wt) of the toxin inactivates elongation factor 2 (EF-2) by catalyzing the covalent attachment of the adenosine diphosphate ribose portion of NAD<sup>+</sup> to EF-2 (see reference 2 and 21 for review). The mechanism by which fragment A is transported from the cell exterior to the cytoplasm is unknown. Theories to explain the transport process suggest either that the toxin forms a pore in the plasma membrane through which fragment A passes (1) or that the toxin is first endocytosed and then somehow escapes to the cytoplasm from an intracellular vesicle (19).

Lysosomotropic drugs, such as ammonium chloride (13) and chloroquine (15), protect cells against the cytotoxic effects of diphtheria toxin. Ammonium chloride does not inhibit the *in vitro* catalytic activity of fragment A (12, 18) nor the adsorption

of the toxin to the cell surface (14). Chloroquine does not inhibit the adsorptive endocytosis of diphtheria toxin, although it was not certain that the toxin molecules observed in this study were those that eventually could reach the cytoplasm (15). The evidence suggests that lysosomotropic drugs block the penetration of fragment A into the cytoplasm sometime after the toxin binds to the cell surface. We investigated the effects of these drugs to better understand how fragment A is transported from the cell exterior to the cytoplasm.

We address two problems in this study. First, it is not clear whether fragment A penetrates the plasma membrane or whether it penetrates the membrane of an intracellular compartment after the toxin is endocytosed. Information about the location of toxin that is blocked during entry by ammonium chloride could help answer this question; if the toxin is trapped at the cell surface, it should be neutralized by antitoxin and have no effect on protein synthesis when the ammonium chloride is removed. If it is within an intracellular vesicle, the

antitoxin should be ineffective when the ammonium chloride is removed. It is well known that the effects of the toxin are neutralized by antitoxin added at 37°C to cells that were preincubated with ammonium chloride and toxin (12, 14). This suggests that the toxin is trapped at the cell surface. However, we considered the possibility that the temperature of the cells at the time of antitoxin addition may have influenced this result. We repeated these experiments but chilled the cells to 4°C during the exposure to antitoxin and found that the effects of the toxin were not inhibited. This suggests that ammonium chloride does not simply trap the toxin at the cell surface.

The second problem we address is the mechanism by which lysosomotropic drugs protect cells against diphtheria toxin. Lysosomotropic drugs raise the pH within acidic intracellular compartments; for example, ammonium chloride raises the pH in macrophage lysosomes from ~4.7 to >6.0 (20). This could be the activity shared by these drugs that arrests the entry of fragment A into the cytoplasm. If so, then exposing the toxin to an acidic environment may be an important step in the penetration process. To test this idea, we used the experimental approach recently described by Helenius et al. (11) in their study of Semliki Forest virus. We exposed cells with diphtheria toxin bound to their surface to acidic medium. If this substituted for the function that was normally inhibited by lysosomotropic drugs, then the drugs might no longer protect cells against the toxin. We found that they did not. Furthermore, antitoxin did not protect the cells after the treatment at low pH.

## MATERIALS AND METHODS

### Materials

Diphtheria toxin (lot D356) was purchased from Connaught Laboratories, Ontario, Canada, and purified by DE-52 chromatography by the general procedure of Collier and Kandel (3). 90% of the toxin was estimated to be intact as determined by polyacrylamide gel electrophoresis with sodium dodecyl sulfate and 2-mercaptoethanol. Fragment A of diphtheria toxin was the gift of Dr. Gary Gilliland and Dr. John Collier, University of California, Los Angeles. Other materials were obtained from the following sources: Ultrogel Aca22, LKB Instruments, Inc., Rockville, Md.; NH<sub>4</sub>Cl, Mallinckrodt, Inc., St. Louis, Mo.; tributylamine, Matheson, Coleman, and Bell, E. Rutherford, N. J.; glutaraldehyde, Polysciences, Inc., Warrington, Pa.; deoxyribonuclease I, Worthington Biochemical Corp., Freehold, N. J.

### Methods

**CELLS AND CULTURE CONDITIONS:** All cells were routinely maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum as previously described (7, 8). V79 cells were originally derived from Chinese hamster lung and were obtained from Dr. Immo Scheffler, University of California, San Diego. VERO cells were originally derived from African green monkey kidney and were obtained from the American Type Culture Collection, Rockville, Md. (registry number CCL81). Cell line Dtx<sup>-</sup>-2 used in the experiment of Fig. 2 is a mutant derived from V79 cells and has a lowered affinity for diphtheria toxin. This is described as a class II mutant in reference 8 and as Dtx<sup>-</sup>-2 in reference 6. Citrate-buffered saline, pH 4.6, contained citrate (0.02 M) and NaCl (0.9 M).

**PROTEIN SYNTHESIS ASSAYS:** The protein synthesis assay for the activity of diphtheria toxin in cultured cells has been described (6). Briefly, cells grown in 24-well Falcon plates containing medium with one-twentieth the normal amount of methionine were exposed to 2  $\mu$ Ci/ml of [<sup>35</sup>S]methionine for 1 h, washed with phosphate-buffered saline, and dissolved in a solution of 0.5% sodium dodecyl sulfate, 1 mg/ml deoxyribonuclease I, 1 mM MgCl<sub>2</sub>, and 1 mM CaCl<sub>2</sub>. The solution of dissolved cells was transferred from the plates directly to squares of Whatman 3 MM paper (Whatman, Inc., Clifton, N. J.), soaked in trichloroacetic acid (10%), washed twice in ethanol (95%), dried, and assayed for radioactivity in a liquid scintillation counter. All assays were done in triplicate. The experimental manipulations required in this work were such that a medium containing CO<sub>2</sub> was undesirable for the assay. Medium A was Dulbecco's modified Eagle's medium with 10% fetal bovine serum buffered to pH 7.0 with

0.02 M HEPES instead of CO<sub>2</sub> and containing one-twentieth the normal amount of methionine.

**ACIDIC MEDIUM:** The acidic medium used in these experiments was prepared by adjusting complete medium A to the desired pH with HCl.

**RABBIT ANTITOXIN:** Diphtheria toxin was inactivated by dialysis for 24 h against 2% formaldehyde, redialyzed against phosphate-buffered saline, mixed with complete Freund's adjuvant, and injected into rabbits on a standard schedule. Affinity-purified antitoxin was prepared by passing antiserum over a column of toxin coupled to Ultrogel Aca22 and eluted with 0.2 M HCl adjusted to pH 2.2 with glycine according to the general procedure of Louvard et al. (16).

## RESULTS

### *The Location of Diphtheria Toxin Blocked by Ammonium Chloride*

Kim and Groman (14) and Ivins et al. (12) found that antitoxin inhibited the cytotoxic effects of diphtheria toxin when cells were preincubated with toxin and ammonium chloride, exposed to antitoxin at 37°C, and then washed to remove the ammonium chloride block. The toxin must have been accessible to the antitoxin; however, this does not demonstrate that the toxin was immobilized at the cell surface. Any active contribution by the cells to the interaction of toxin and antitoxin at 37°C was not controlled in these experiments. We repeated this study and reproduced the results of Ivins et al. (12) when cells were exposed to antitoxin at 37°C (Table I, column I); however, when the cells were exposed to antitoxin at 4°C, fragment A later entered the cytoplasm to inhibit protein synthesis (Table I, column II).

We tried similar experiments with other lysosomotropic drugs instead of ammonium chloride. Chloroquine was unsuitable because its effects were not completely reversed when the cells were washed. Tributylamine, however, was an effective and reversible inhibitor of the toxin. In the presence of 5 mM tributylamine, the toxin again entered an antitoxin-insensitive condition if the antitoxin was added to cells at 4°C (data not shown). This suggests that the observation may be generalized to other lysosomotropic drugs whose toxin-inhibitory activity can be reversed.

### *The Effect of an Acidic Environment on Diphtheria Toxin that is Bound to the Cell Surface*

Lysosomotropic drugs raise the pH within acidic intracellular compartments (4, 20). The effect of these drugs on the cytotoxic activity of diphtheria toxin could be explained if exposing the toxin to an acidic environment (perhaps within lysosomes) were necessary for fragment A to enter the cytoplasm. We simulated acidic conditions which the toxin might encounter at some step during the entry process by exposing cells with toxin on their surface to acidic medium. We then measured the effects of lysosomotropic drugs on the activity of the toxin to see if the acidic environment could substitute the function normally inhibited by the drugs. A representative experiment is shown in Fig. 1. V79 cells were incubated with toxin in medium A, pH 7.0, for 1 h at 4°C and washed to remove excess toxin. The cells were then exposed to medium A at different acidities between pH 7.0 and 4.0 at 4°C for 0.5 h. The time of exposure was determined from preliminary experiments. The medium was then replaced with medium A, pH 7.0, containing 10 mM ammonium chloride, and the cells were incubated for an additional 0.5 h at 4°C to allow the ammonium chloride time to equilibrate with the cells. The temperature was finally raised to 37°C and protein synthesis was measured 3 h later.

TABLE I

The Effect of Temperature on the Ability of Antitoxin to Inhibit Diphtheria Toxin that was Incubated with Cells in the Presence of Ammonium Chloride

Diphtheria toxin	Protein synthesis (cpm $\pm$ SD $\times 10^{-3}$ )	
	I	II
	Antitoxin	Antitoxin
M	37 °C	4 °C
0	35 $\pm$ 3	34 $\pm$ 1
10 <sup>-11</sup>	37 $\pm$ 1	38 $\pm$ 2
10 <sup>-10</sup>	39 $\pm$ 4	39 $\pm$ 1
10 <sup>-9</sup>	35 $\pm$ 1	5.5 $\pm$ 0.5
10 <sup>-8</sup>	35 $\pm$ 1	1.1 $\pm$ 0.1
10 <sup>-7</sup>	34 $\pm$ 1	0.8 $\pm$ 0.2

V79 cells were incubated with 10 mM ammonium chloride in medium A at 37°C for 15 min and then exposed to the indicated concentrations of diphtheria toxin at 37°C for 1 h in the presence of the ammonium chloride. Antitoxin (a 1:20 dilution of antiserum) was added for 0.5 h to the cultures in column I at 37°C and to the cultures in column II at 4°C. All the cells were then chilled to 4°C and washed four times during the next hour with medium A lacking ammonium chloride. After the last wash, the cells were incubated at 37°C for 24 h, during the last hour of which they received [<sup>35</sup>S]methionine.

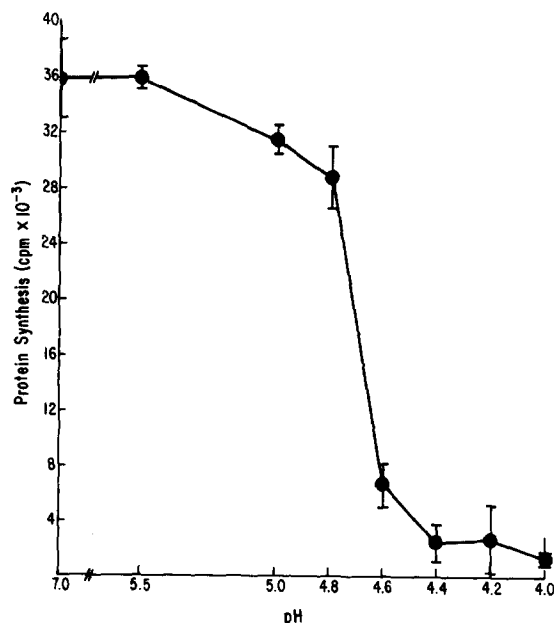


FIGURE 1 The effect of ammonium chloride on the cytotoxic activity of diphtheria toxin after toxin bound to the cell surface was exposed to acidic medium. V79 cells were exposed to medium A containing 20 nM toxin for 1 h at 4°C, washed three times to remove excess toxin, and treated with medium A at the desired pH for 0.5 h at 4°C. The cells then were incubated with medium A at pH 7.0 containing NH<sub>4</sub>Cl (10 mM) at 4°C for 0.5 h after which time the cells were warmed to 37°C and pulsed with [<sup>35</sup>S]methionine during the last hour of a 3-h incubation. When NH<sub>4</sub>Cl was withheld from cells treated at pH 7.0, there were 2.5  $\pm$  0.3 cpm  $\times 10^{-3}$  in the trichloroacetic acid-insoluble material. Treating the cells with medium at a low pH in the absence of toxin had little effect on protein synthesis.

The results in Fig. 1 show that the ammonium chloride failed to protect cells that were exposed to a pH below 4.7. We obtained similar results when the lysosomotropic agent was tributylamine or chloroquine (data not shown). Citrate- or acetate-buffered saline gave the same results as the medium at a low pH, indicating that none of the normal components of the medium were required for the effect.

This result could be explained if exposing the cells to acidic medium damaged their plasma membranes so that the toxin diffused into the cytoplasm. Isolated fragment A of diphtheria toxin is not cytotoxic at moderate concentrations because it does not penetrate intact membranes. We treated cells with medium at pH 4.0 in the presence of fragment A to test for membrane damage. As shown in Table II, fragment A did not inhibit protein synthesis, indicating that the plasma membrane barrier of the cells was intact. Our results could also be explained if ammonium chloride temporarily lost the potential to interact with cells after they were exposed to a low pH. We tested this by measuring the effect of ammonium chloride on the toxin with cells that were previously exposed to acidic medium in the absence of the toxin. As a control for this experiment, we determined that the cells were still sensitive to the toxin after an acidic treatment in the absence of the toxin (Table III, column II). Thus, the acidic medium did not remove the toxin receptor or otherwise impair the ability of the cells to transport fragment A into their cytoplasm. When ammonium chloride was included in the medium with the toxin, the cells were protected, indicating that the acidic medium did not do something to the cells that rendered the ammonium chloride ineffective (Table III, column III).

TABLE II

The Effect of Fragment A on Protein Synthesis in the Presence of Acidic Medium

Fragment A	pH of medium during treatment	Protein synthesis
		cpm $\pm$ SD $\times 10^{-3}$
-	7.0	24 $\pm$ 1
+	7.0	26 $\pm$ 1
-	5.0	24 $\pm$ 1
+	5.0	23 $\pm$ 1
-	4.0	26 $\pm$ 1
+	4.0	24 $\pm$ 3

V79 cells were incubated with medium A at different acidities containing either 10  $\mu$ g/ml fragment A or no fragment A for 0.5 h at 4°C. The medium was replaced with medium A at pH 7.0 with or without fragment A and containing 10 mM NH<sub>4</sub>Cl. 0.5 h later, the cells were warmed to 37°C and pulse labeled during the last hour of a 3-h incubation.

TABLE III

The Effect of an Acidic Environment on the Sensitivity of V79 Cells to Diphtheria Toxin and on the Ability of Ammonium Chloride to Inhibit the Toxin

Diphtheria toxin	Protein synthesis (cpm $\pm$ SD $\times 10^{-3}$ )		
	I	II	III
	pH 7 -NH <sub>4</sub> Cl	pH 4 -NH <sub>4</sub> Cl	pH 4 +NH <sub>4</sub> Cl
M			
0	40 $\pm$ 6	38 $\pm$ 3	37 $\pm$ 2
10 <sup>-10</sup>	42 $\pm$ 2	40 $\pm$ 3	40 $\pm$ 2
10 <sup>-9</sup>	30 $\pm$ 1	24 $\pm$ 3	36 $\pm$ 2
10 <sup>-8</sup>	4.1 $\pm$ 0.4	3.8 $\pm$ 0.5	38 $\pm$ 1
10 <sup>-7</sup>	1.2 $\pm$ 0.3	1.4 $\pm$ 0.5	38 $\pm$ 1

The cells in columns II and III were incubated at 4°C with medium A at pH 4.0 for 0.5 h in the absence of toxin. The cells in column I were treated the same, except that the medium was at pH 7.0. All cells were then incubated with medium A at pH 7.0 containing the indicated concentrations of toxin at 4°C for 1 h and washed to remove the excess toxin. The cells in column III were then incubated at 4°C with medium A containing 10 mM ammonium chloride at 4°C for 0.5 h while the cells in columns I and II were treated the same, except that the medium A had no ammonium chloride. All the cells were warmed to 37°C without a medium change and pulsed with [<sup>35</sup>S]-methionine during the last hour of a 3-h incubation.

The first step in the normal intoxication process is binding of diphtheria toxin to a cell surface receptor. To see if this same receptor was required for intoxication after treatment at a low pH, we compared the dose-dependent response of V79 cells with the dose-dependent response of mutant Dtx<sup>-</sup>2 cells. The mutant cells have an apparent lower affinity for the toxin than wild-type cells (8). This difference is reflected in a shift of the dose-response curve to higher toxin concentrations. This is shown at the top of Fig. 2. The cells were incubated with the toxin at 4°C for 3 h and washed. They were then incubated at 4°C for 0.5 h without toxin before the temperature was raised to 37°C. The rate of protein synthesis was measured 3 h later. In parallel, as illustrated in the lower panel of Fig. 2, the cells were incubated with toxin at 4°C for 3 h, washed, and then exposed to medium at a low pH for 0.5 h. This medium was replaced with medium A at 37°C containing 10 mM ammonium chloride, pH 7.0, and protein synthesis was measured 3 h later. The shift in the dose-response curve was still present, suggesting that protein synthesis was arrested by toxin that was originally associated with the toxin receptor.

The antigenic determinants of diphtheria toxin at the cell

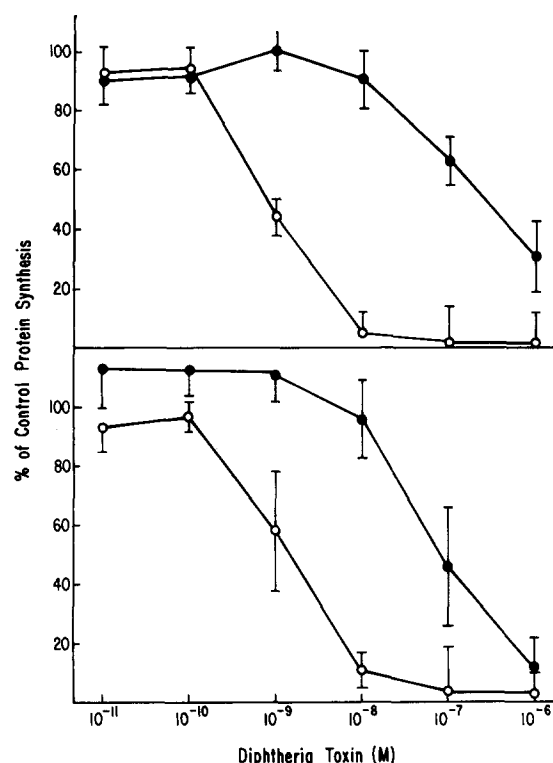


FIGURE 2 The normal dose-response of V79 and Dtx<sup>-</sup>2 cells to diphtheria toxin (top) and the dose-response in the presence of ammonium chloride after toxin was exposed to acidic medium at the cell surface (bottom). Top, wild-type V79 cells (○) and mutant Dtx<sup>-</sup>2 cells (●) were exposed to diphtheria toxin for 3 h at 4°C, washed three times, warmed to 37°C, and pulsed with [<sup>35</sup>S]methionine for the last hour of a 3-h incubation. In the absence of diphtheria toxin, there were  $41 \pm 2 \text{ cpm} \times 10^{-3}$  in the V79 samples and  $34 \pm 2 \text{ cpm} \times 10^{-3}$  in the Dtx<sup>-</sup>2 samples. Bottom, wild-type V79 cells (○) and Dtx<sup>-</sup>2 cells (●) were exposed to diphtheria toxin for 3 h at 4°C, washed three times, exposed to medium A at pH 4.0 for 0.5 h at 4°C followed by incubation with medium A at pH 7.0 containing NH<sub>4</sub>Cl (10 mM) for 0.5 h. The cells were then warmed to 37°C and pulsed with [<sup>35</sup>S]methionine for the last hour of a 3-h incubation. In the absence of toxin there were  $49 \pm 1 \text{ cpm} \times 10^{-3}$  in the V79 samples and  $19 \pm 2 \text{ cpm} \times 10^{-3}$  in the Dtx<sup>-</sup>2 samples.

surface might be altered after the exposure to acidic medium. Any change could reflect some step of the process by which fragment A enters the cytoplasm. We, therefore, exposed cells with toxin on their surface to antitoxin either before or after treatment at low pH. The antitoxin was added to cells at neutral pH and at 4°C in this experiment to minimize any effects of pH or of cellular activity on the interaction of toxin and antitoxin. When the antitoxin was added before the low pH treatment, the toxin had no effect on protein synthesis (Table IV, column II). This demonstrated that the antitoxin was active. However, when the antitoxin was added after the low pH treatment, the toxin inhibited protein synthesis (Table IV, column III). The acidic environment triggered some event that either changed or sheltered the antigenic determinants of the toxin.

## DISCUSSION

If lysosomotropic drugs trap diphtheria toxin within an intracellular vesicle, then it should be possible to segregate the toxin from antitoxin added to the culture medium. Kim and Groman (12) and Ivins et al. (14) found no segregation when the antitoxin was added to cells at 37°C; however, when we chilled cells to 4°C before the antitoxin was added, the toxin subsequently proceeded to inhibit protein synthesis. The simplest interpretation is that lysosomotropic amines trap the toxin within a vesicle whose contents are accessible to antitoxin at 37°C but not at 4°C. This could occur if the toxin were endocytosed and delivered to secondary lysosomes from which it could not escape. Antitoxin could be later delivered to the same compartment after fluid endocytosis. There is a precedent for this: Gordon et al. (10) observed that macromolecules added to cells at different times could be found in the same secondary lysosome. Another possibility is that lysosomotropic drugs upset the vesicle traffic in the cell so that the toxin is recycled back to the cell exterior after endocytosis. Both mechanisms would separate the toxin and antitoxin at 4°C when the cells are quiescent. Both mechanisms also imply that fragment A escapes to the cytoplasm from an intracellular vesicle after endocytosis.

TABLE IV  
The Effect of Antitoxin on the Cytotoxic Activity of Diphtheria Toxin before and after Toxin Bound to the Cell Surface was Exposed to Acidic Medium

Diphtheria toxin	Protein synthesis (cpm $\pm$ SD $\times 10^{-3}$ )		
	I No antitoxin	II Antitoxin before low pH treatment	III Antitoxin after low pH treatment
5 nM			
+	$2.7 \pm 0.3$	$26 \pm 1$	$1.3 \pm 0.1$
-	$24 \pm 5$	$26 \pm 1$	not measured

There were five basic steps in the experimental protocol. (a) VERO cells were incubated in medium A at pH 7.0 with or without toxin at 4°C for 0.5 h. (b) The medium was changed to medium A without antitoxin in columns I and III and with antitoxin in column II. The cells were then incubated at 4°C for 0.5 h. (c) All cells were incubated with 0.02 M citrate-buffered saline, pH 4.6, for 0.5 h at 4°C. (d) This was changed to medium A at pH 7.0 containing 10 mM ammonium chloride without antitoxin in columns I and II and with antitoxin in column III. The cells were then incubated at 4°C for 0.5 h. (e) The cells were washed twice with medium A at pH 7.0 containing 10 mM ammonium chloride and incubated with this medium at 37°C for 3 h, during the last hour of which they received [<sup>35</sup>S]methionine. The antitoxin used in the incubations was affinity purified and the concentration was 10  $\mu\text{g/ml}$ .

There is another interpretation of these data. Lysosomotropic drugs could trap the toxin at the cell surface but the act of chilling the cells may nevertheless prevent the interaction of toxin with antitoxin. This could happen if the toxin were endocytosed in response to the temperature drop or if the structure of the toxin changed at the low temperature. The former possibility still implies that fragment A enters the cytoplasm from a vesicle. It is not likely that the structure of toxin is altered at 4°C; the toxin binds to the cell surface at 4°C and its antigenic determinants are intact because antitoxin added at 4°C and then washed away protects the cells.

Because lysosomotropic amines neutralize acidic intracellular pH gradients, they could trap diphtheria toxin within a vesicle, such as a lysosome, if an acidic environment were necessary for fragment A to penetrate a membrane. We, therefore, studied the consequences of exposing diphtheria toxin on the cell surface to an acidic environment. Lysosomotropic drugs fail to protect cells from the toxin after this treatment. An important question is whether this phenomenon is related to the normal process by which fragment A enters the cytoplasm. The acidic treatment could induce fragment A to enter the cytoplasm by a mechanism fundamentally different than normal and, therefore, insensitive to normal inhibitors. We cannot disprove this possibility; however, the result correlates better with other data if the acidic environment provides the step that lysosomotropic drugs normally inhibit. There is ample evidence that diphtheria toxin could have access to the acidic intralysosomal environment. Toxin bound to the surface of VERO cells is endocytosed and enters the lysosomes, although the toxin concentration in these studies was high and it is not known if toxin derived from the lysosomal pool eventually inhibits protein synthesis (5, 15, 17). Our evidence suggests that fragment A can enter the cytoplasm from intracellular vesicles, implying that endocytosis occurred at low toxin concentrations, but the identity of the vesicles is unknown. Many other cell-surface-associated protein ligands are delivered to lysosomes after endocytosis (9). We do not suggest that the only place the toxin could encounter an acidic environment is within lysosomes, but the influence of a low pH on the toxin should be the same in lysosomes as it is at the cell surface. Lysosomotropic drugs would trap the toxin within lysosomes if they prevented the step that committed fragment A to enter the cytoplasm by neutralizing the low pH. They would not inhibit if exposing the toxin to an acidic environment at the cell surface had already committed fragment A to enter the cytoplasm. The correlation existing among the acidic intralysosomal environment, the effects of lysosomotropic amines on this environment, and the effects of an acidic environment on toxin at the cell surface is probably more than coincidence.

Antitoxin has no effect on the toxin after the toxin is exposed to a low pH at the cell surface. The toxin either undergoes a conformational change or is sheltered from the antitoxin or both. This could occur if the acidic conditions caused the endocytosis of the toxin at 4°C. There is evidence against this; the cells are still sensitive to the toxin after they are exposed to acidic medium in the absence of the toxin. This implies that the toxin receptor is not removed from the surface at 4°C under these conditions. It is, therefore, unlikely that toxin bound to this receptor at 4°C is removed. The failure of the antibody to interact with the toxin probably reflects a functional change in the structure of the toxin that is required to transfer fragment A through a membrane. Boquet et al. (1) suggested that diphtheria toxin, or a proteolytic fragment of

the toxin, inserts into the plasma membrane to translocate fragment A through the membrane. Donovan et al. recently found that intact diphtheria toxin forms transmembrane channels in planar lipid bilayers, but not until the pH is lowered.<sup>1</sup> Direct evidence that fragment A penetrates the bilayer when the toxin inserts is lacking, but it is a reasonable possibility. It is likely that when toxin at the cell surface is exposed to a low pH, it either inserts into the membrane or is conformationally altered to expose an hydrophobic domain in preparation for insertion. Loss of original antigenic sites on the toxin during this process is expected. Fragment A could pass through the plasma membrane at 4°C or at some later time after the temperature is raised to 37°C. In either case, the cells would be committed to intoxication beyond rescue by antitoxin or by lysosomotropic agents. Similar events could occur when the toxin encounters an acidic environment within lysosomes.

The evidence presented here supports the following mechanism for the normal entry of fragment A into the cytoplasm: (a) association of diphtheria toxin with a receptor, (b) endocytosis, (c) acidification of the vesicle interior, possibly by fusion with a lysosome, and (d) insertion of the toxin into the vesicle membrane in response to the acidic environment. Fragment A is presumably transferred to the cytoplasmic side of the membrane during insertion. This mechanism is similar to that proposed by Helenius et al. (11) for the infection of BHK21 cells by Semliki Forest virus, except that the viral membrane is believed to fuse with the lysosomal membrane in response to the low pH. A similar mechanism may operate to transfer other biologically relevant proteins into the cell cytoplasm.

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